

DENSITY GRADIENT STUDIES OF BACTERIOPHAGE T3

by

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INTRODUCTION

The past fifteen years have been witness to a rebirth of research involving bacterial viruses. Reasons for this renewed interest in bacteriophages are multiple. The relatively simple structure of these particles and the ease with which they are propagated and purified make them ideal subjects for investigations in several fields. For the student of genetics, the bacteriophage is an excellent organism since it can produce thousands of offspring within a few hours and participates in genetic interactions which are well suited for quantitative genetic analysis. The bacteriophage, being easily handled experimentally, provides the virologist with a model organism for explorations concerning the many activities involved in intracellular parasitism. Molecular biologists concerned with the replication and properties of nucleic acid have recognized the bacteriophage as an invaluable tool. In addition, the enzymologist, immunologist, epidemiologist, medical microbiologist, and investigators in many other fields have successfully utilized the bacteriophage in their researches.

A recently described phenomenon of genetic interest involves bacteriophage active against Escherichia coli. Variants of phage T3 have been reported that differ with regard to antigenic, morphological, and host range properties (Eisenstark, Maaløe and Birch-Andersen, 1961). One of these T3 variants, designated T3B, elicits the production of antibodies capable of inactivating T3B phages, but incapable of inactivating another T3 variant designated T3C. The T3C particle, however, elicits antibodies capable of inactivating both T3B and T3C.

The morphological distinction between the two variant phages involves the tail structure: T3C has a flexible tail approximately $2\frac{1}{2}$ head diameters long, while T3B has a short tail about one-half head diameter in length.

The two variants differ in host range and this difference has been utilized in determining the numbers of each type of phage in mixed populations. T3B can be propagated on E. coli strains B, B/1 and C600. T3C plates on E. coli strains B and HfrC.

The antigenic, morphological, and host range differences in these two T3 variants are of even greater interest in light of another observation reported by Eisenstark and coworkers. When samples are taken from individual plaques of T3B, particles of the T3C type are regularly found at a frequency of about 10^{-6} . In contrast, when samples are taken from individual plaques of T3C, the frequency of T3B type particles is extremely low, reported as probably less than 10^{-11} . These authors consider several possibilities to account for the stability of T3C phages and the frequent appearance of T3C phages in T3B stocks:

(1) The change from T3B to T3C might involve a deletion of genetic material.

(2) The change from T3B to T3C might involve a point mutation with a normal frequency of about 10^{-6} .

(3) Possibly T3B is a point mutation of T3C, with an extremely low frequency of less than 10^{-11} . In this case the change from T3B to T3C would be a reverse mutation.

(4) The mutation from T3B to T3C might involve a suppressor gene which now permits the synthesis of the new tail structure.

(5) The "code" for the new tail structure may not be included in the T3B genome at all; rather, this "code" may reside in the genome of the host cell and be picked up by rare phage as a result of phage-host interaction.

The experiments described in this thesis constitute an effort to determine, by ultracentrifugal methods, whether the two T3 variants differ in respects not previously recognized, and to consider the sum differences and similarities in an attempt to elucidate the actual genetic mechanism involved in the described phenotypic alterations.

The Review of Literature includes an extensive discussion of the density gradient procedures since the experiments in this thesis involve the use of this technique.

REVIEW OF LITERATURE

Development of Density Gradient Techniques

Centrifugation is widely recognized as one of the most useful methods employed in the isolation, purification, and critical examination of biological materials. Of particular value have been results obtained with the analytical ultracentrifuge in determining the size, shape, density, and molecular weight of such biological materials as cell particulates, viruses, proteins, and nucleic acids. This instrument, however, is not available to every investigator. Recent studies have been directed toward the development of techniques whereby the less complicated, and less expensive, preparative centrifuge can be employed in experiments formerly limited to the analytical centrifuge.

The basic difference between the analytical and the preparative

centrifuge is the presence, on the analytical instrument, of an optical system. This enables one to follow the migrating particle during the actual centrifugal process. The lack of an optical system on the preparative centrifuge necessitates curtailment of the centrifugal process before the migrating particles can be studied. Serious disturbances result during deceleration and migrating boundaries are disrupted.

Another common difference observed concerns the geometry of the tubes and rotors. The analytical instrument is normally equipped with sector-shaped tubes in a swinging bucket rotor, so that the tubes are in a horizontal position during the operation of the instrument. In contrast, the preparative centrifuge is ordinarily equipped with parallel-walled tubes held in an angle-head rotor. Serious convective disturbances occur in such angle-head rotors.

Thus, if the preparative centrifuge is to be employed in analytical studies, two distinct conditions must be satisfied:

- (1) A method must be employed to minimize disturbances in the tube, both during rotation at the maximum speed and during the deceleration process, so that boundaries of migrating particles can be formed and maintained.
- (2) A technique must be available to locate the boundary of migrating particles.

Once the first condition is satisfied, the second is seldom a hazard.

Pickels (1943) performed a detailed study of particle sedimentation in tubes inclined at an angle to the axis of rotation, and interpreted the resulting disturbances as follows: particles start to sediment normally, in accordance with the magnitude of the field and properties of

the molecules and the solvent. However, normal migration does not continue due to forces which arise as a consequence of the tube's inclined position. The radial movement of particles away from the axis of rotation causes a clearing of the fluid along the inner wall of the tube, and an accumulation of particles against the outer wall of the tube. Thus density increases at the outer wall and decreases along the inner wall of the tube, assuming the density of the sedimenting particle is greater than that of the solvent. Such a system attempts to adjust itself; liquid along the outer wall descends, while liquid along the inner wall ascends. This movement results in the transport of particles to the bottom of the tube, where they remain, at a rate more rapid than normal. Such an explanation accounts for the fact that angle-head rotors are valuable for rapid sedimentation. This explanation also indicates why distinct boundaries of migrating particles are not formed or maintained in the angle-head rotor, and one realizes that the preparative centrifuge is useless in analytical experiments unless preventive measures are enacted.

Pickels found that the convective disturbances of inertial origin discussed above, and also disturbances of thermal origin, may be counteracted by furnishing the material under study with a synthetic density gradient, formed with sucrose or some other non-sedimentable material. With such a density gradient, the greater density would always be farther from the axis of rotation and for convection to occur denser liquid would have to flow upward and push lighter liquid downward. This tendency is counteracted by the centrifugal field. This development was very valuable in the separation of cellular components, but was not too satisfactory in analytical studies.

The results of Kahler and Lloyd (1951a) in their studies on the sedimentation rate of polystyrene latex, indicated that with light particles or dilute solutions convections are not sufficiently prevented by the use of a density gradient. These workers employed parallel-walled tubes, but devised a rotor containing buckets for the tubes which would pivot from vertical to horizontal, so that the direction of centrifugal force would always be parallel to the walls of the tubes. They conclude that such a swinging-bucket rotor has much advantage over the angle-head rotor, but that enough convection remained to necessitate the use of a density gradient. Using a preparative centrifuge with a swinging-bucket rotor and glycerin to form a stabilizing gradient, Kahler and Lloyd arrived at a value for the sedimentation coefficient of polystyrene latex that was in excellent agreement with the value obtained by using the analytical ultracentrifuge.

Hogeboom and Kuff (1954) extended the use of the swinging-bucket rotor to studies of various proteins and enzymes, and found that sedimentation constants ranging between 4 and 2000 Svedberg units were in close agreement with values obtained in the analytical instrument. The materials investigated by these workers ranged from molecules as small as lysozyme and serum albumin to particles as large as polystyrene latex. A sucrose gradient was employed in these studies.

In common usage the term "density gradient centrifugation" is employed to describe several different procedures. Anderson (1955a) points out that two basic principles are utilized in the centrifugal separation and characterization of particles. The first of these, differential centrifugation, depends on differences in sedimentation rates. The second

principle, isopycnic centrifugation, is based on differences in density. Since density gradients may be used with either principle, Anderson has proposed the following classifications:

(1) Differential centrifugation. In this method, fractions are collected consecutively at the bottom of the tube. The separation achieved depends on differences in sedimentation rates.

(2) Gradient differential centrifugation. Particles are separated according to their sedimentation rate by centrifuging them through a density gradient which prevents convection currents and other anomalous effects. Fractions may be collected at the bottom, top, or anywhere along the gradient.

(3) Isopycnic centrifugation. Separation is achieved by centrifuging the particles in a solution having the same density as the fraction desired. Fractionation, therefore, depends on particle density alone. Centrifugation is continued until denser or lighter particles have been sedimented to the bottom or floated to the top of the tube.

(4) Isopycnic gradient centrifugation. This type of separation is based on the technique described by Linderström-Lang (1937) for determining the densities of microdrops of liquid. Particles are separated by sedimenting them through a density gradient until each species finds its isopycnic level. Centrifugation is continued until this equilibrium condition is established. It is evident that the separation observed before the equilibrium is established will depend upon sedimentation rate, while the separation observed after establishment of equilibrium depends only on particle density.

Density gradients may be formed by a variety of methods. Sucrose has been the most widely used material although its high viscosity has been a disadvantage. A simple method of producing a sucrose gradient is to layer successive sucrose solutions of decreasing density into a centrifuge tube and allow diffusion to eliminate the discontinuities which exist at the interfaces. Anderson (1955b) has described a mechanical device for the rapid production of a smooth gradient which is accurately reproducible. Such a machine can produce gradients of varied shapes which may aid in the separation procedure.

The experiments of Meselson, Stahl and Vinograd (1957) set forth a new version of gradient formation. In prior experiments the gradient employed was preformed by some type of layering technique. These investigators formed the density gradient during the centrifugation process. The density gradient is established by the sedimentation of a low molecular weight solute, such as cesium chloride, rubidium chloride, or sodium bromide in a solution subject to a constant centrifugal field. The solution is centrifuged until equilibrium is approached. The opposing tendencies of sedimentation and diffusion have then produced a stable concentration of the solute, the centrifugal field strength, and the length of the liquid column may be chosen so that the range of density at equilibrium encompasses the effective density of the sedimenting particle. The centrifugal field tends to drive the particle into the region where the sum of the forces acting on a given particle is zero. The density of the solution in this region is equal to the effective density

of the particle. Meselson and coworkers also derived equations for calculating molecular weights of molecules from measurements of the position and shape of the band formed, as well as the properties of the material forming the gradient. Baldwin (1959) has shown that heterogeneity among the molecules can cause serious errors in molecular weights calculated by this method. Sueoka (1959), Yeandle (1959), and Trautman (1960) report theoretical studies concerning interactions between molecules and either the solvent or gradient material which alter molecular weight values calculated by this technique.

In addition to sucrose and the inorganic salts discussed, several other substances have been employed to form density gradients. Ficoll, a water soluble neutral colloid with properties similar to a polysaccharide, was employed by Holter and Møller (1958). Additional compounds employed include a solution of heavy water and sucrose (Kahler et al., 1954), hemocyanin (Polson and Madsen, 1954), colloidal thorium oxide (de Duve et al., 1959), glycerol (Kahler and Lloyd, 1951b), potato starch (Anderson, 1958), and polyvinylpyrrolidone (Thomson and Klipfel, 1958).

Application of Density Gradient Techniques to Studies of Viruses and Nucleic Acids

Biologists quickly recognized the potential of the density gradient technique. Friedewald and Pickels (1944) employed the technique described by Pickels (1943) in a study of the PR8 and Lee strains of influenza virus. These investigators, using an angle head centrifuge and a sucrose gradient, observed sedimenting boundaries of infective particles, hemagglutinin, and complement-fixing antigen which correlated with boundaries observed optically in the ultracentrifuge. Particle size estimates by these workers,

calculated from angle head centrifugal data, agreed with values obtained by filtration with graded collodion membranes.

Brakke (1951, 1953 and 1956) studied the potato yellow-dwarf virus using a sucrose gradient and observed that this method of purification resulted in a marked stabilization of infectiousness and morphology. Various chemicals were also added to the virus sample, followed by purification in a sucrose gradient, to determine the value of the chemicals as stabilizing agents for the virus.

When partially purified preparations of wild cucumber mosaic virus are examined in an ultracentrifuge, two schlieren peaks are observed (Sinclair et al., 1957). Such preparations, when centrifuged in a sucrose gradient, produced two opaque layers. These workers found the upper layer was non-infectious whereas the lower layer was infectious. Morphologically, particles from the two layers are similar in size and shape. Noting that the top component absorbs less at $260\text{ m}\mu$, Sinclair and his coworkers suggest that the top component is a nucleic-acid free protein similar in structure to the protein portion of the intact virus.

Wheat streak mosaic virus is present in the host plant in low concentration and is easily inactivated (Brakke, 1958a). Brakke utilized a sucrose gradient to purify the virus and study agents which affect its stability. This purification procedure was employed by Moorhead (1959) to prepare antigens for a serological analysis of wheat streak mosaic virus. Brakke and Staples (1958), during sucrose gradient purification of wheat streak mosaic virus, noted that samples taken from the visible zone of a gradient were 10-1000 times more infective and contained correspondingly more rods longer than $560\text{ m}\mu$ than did samples elsewhere in the gradient.

Brakke (1953b) describes a new method of calculating the sedimentation coefficients of unknown viruses using a sucrose gradient. His results indicate that the ratio of sedimentation rates of two viruses is equal to the inverse of the ratio of times required for the two viruses to sediment from the meniscus to a given depth. This relation holds through small increments of distance. Using tobacco mosaic virus, whose sedimentation coefficient has been determined by other methods, as a reference particle, Brakke has reported the sedimentation coefficients of other viral particles.

Brakke (1959) concentrated barley stripe mosaic virus in a sucrose gradient. This virus, unpurified, aggregates at 40°C in one hour. By determining the amount of infectivity in the virus zone after density gradient centrifugation, the dispersive action of several detergents was assessed.

To separate two complement-fixing particles of foot-and-mouth disease virus, Trautman et al. (1959) used gradient differential centrifugation. This provided the two antigens free from each other for future experiments. These workers also determined the sedimentation rate of the smaller complement-fixing particle. Breese et al. (1960) used this information in designing a method to centrifugally separate the foot-and-mouth disease virus particles from contaminating host material of higher and lower molecular weights.

Using hemocyanin to produce a density gradient, Polson and Madsen (1954) isolated two particles of different sizes from preparations of African horsesickness virus. They found both particles to be infective following the isolation procedure. Polson et al. (1958) also investigated the antigenic composition of poliomyelitis virus employing a hemocyanin gradient.

While observing the migration of alfalfa mosaic virus in the analytical ultracentrifuge, Bancroft and Kaesberg (1960) noted three peaks with sedimentation coefficients of 73S, 89S, and 99S. Using conventional methods such as ammonium sulfate fractionation, calcium phosphate adsorption, and pH adjustment these workers were unable to separate the three fractions. By use of a sucrose gradient technique the three fractions were successfully separated, and the 99S component was demonstrated as being the infective component.

A very important study concerning the replication of deoxyribonucleic acid was performed by Meselson and Stahl (1958). These workers employed cesium chloride in the isopycnic gradient technique to follow the fate of parental DNA molecules. The bouyant density of DNA varies directly with the amount of N^{15} it contains. By growing bacterial cells in a medium containing N^{15} , then transferring these labeled cells to a medium containing N^{14} and centrifuging DNA samples from the cells at various time intervals, impressive results were obtained. Until one generation time elapsed, half-labeled molecules (molecules intermediate in density between N^{15} DNA and N^{14} DNA) accumulate, while fully labeled DNA is depleted. One generation time after adding N^{14} these half-labeled or "hybrid" molecules alone are observed. Two generation times after the addition of N^{14} half-labeled and unlabeled DNA are present in equal amount. Meselson and Stahl conclude that the N^{15} of a DNA molecule is divided equally between two subunits which remain intact through many generations, and that following replication, each daughter molecule has received one parental subunit. Their experimental results are in exact accord with the Watson-Crick hypothesis of DNA structure.

Meselson and Weigle (1961) investigated the mechanism of genetic recombination in bacteriophage λ . Isopycnic density gradient centrifugation was used to determine the distribution of labeled parental DNA among both parent and recombinant genotypes emerging from a two-factor cross between unlabeled phage λ and λ heavily labeled with the isotopes C^{13} and N^{15} . Their results demonstrate the presence of quantities of original parental DNA in recombinant phages. From their studies these workers suggest that the DNA complement of phage λ is contained in a single semiconservatively replicating chromosome; that genetic recombination can occur by chromosome breakage, both chromosomal subunits being broken during recombination; that distances along the genetic map are proportional to the amounts of DNA in the phage chromosome; and that the phage chromosome need not replicate in order to undergo recombination by breakage.

Davern and Meselson (1960) used isopycnic gradient centrifugation to investigate the nature of ribonucleic acid replication. Escherichia coli cells labeled with C^{13} and N^{15} were transferred to a medium containing light isotopes alone. RNA from such cells was centrifuged at varying time intervals after transfer to the light medium. In contrast to the results obtained with DNA, no partially labeled RNA molecules were observed. Fully labeled molecules persisted for at least three generations while unlabeled molecules were being synthesized. These authors conclude that the precursors from which any given RNA molecule is synthesized are drawn from the medium, and that RNA molecules do not participate in extensive exchange reactions either with precursors or among themselves.

A most interesting application of the isopycnic gradient centrifuga-

gation method was reported by Weigle et al. (1959). Using N^{15} labeled phage λ as a heavy reference, these workers simultaneously centrifuged normal phage λ and its defective variant λ dg in a cesium chloride gradient. In such an experiment three distinct ultraviolet absorbing peaks were observed. Two peaks, one composed of the N^{15} labeled λ and one of normal λ , maintained constant density in all experiments. The third peak varied in density, being more dense than normal λ in some experiments and less dense in others. This peak was identified as λ dg. Preparations of λ dg varying in density had independent origin. From their data these workers postulate that normal λ loses part of its chromosome and acquires a variable amount of genetic material from its host bacterium. This interpretation is supported by transduction studies involving λ and λ dg, and also by genetic crosses between the two phages.

Sinsheimer (1959a), in his studies of the very small bacterial virus ϕ X174, observed that a suspension of this virus migrated in the ultracentrifuge as two components, a 114S component and a larger, slower component of 70S. These two components could not be separated by ammonium sulphate fractionation or by electrophoresis, but separation was achieved by using a rubidium chloride density gradient. The 114S component is infective virus and has a density of 1.40, while the larger, slower 70S component has a density of 1.32 and is not infective. The two components have similar appearance in electron micrographs. Sinsheimer suggests that the 70S component represents the protein "coat" of the virus condensed about fragments of host deoxyribonucleic acid (DNA). The DNA contents of the two components are consistent with their observed density differences. In another study (Sinsheimer, 1959b) it was determined that phage ϕ X174 contains a single-stranded DNA. This single-stranded DNA bands in a

cesium chloride density gradient at a density of 1.72, distinctly higher than the density of native Escherichia coli DNA.

Kozinski and Szybalski (1959) used a cesium chloride gradient to investigate the fate of DNA molecules of phage ϕ X174. Phage labeled with 5-bromodeoxyuridine (5BU) were observed to be more dense than unlabeled phage, and separation of the labeled (heavy) and non-labeled (light) phage in a density gradient was successful. Labeled (5BU) phage also show increased sensitivity to ultraviolet light. When bacteria grown on 5BU free medium were infected with 5BU labeled phage, no heavy phages were detected in the progeny by density gradient centrifugation. Similarly no ultraviolet resistant light phages could be found among early phage progeny released by 5BU labeled bacteria infected with unlabeled phage. The authors interpret these results as being indicative of a dispersive mode of DNA replication in phage ϕ X174; this is in contrast to conservative or semi-conservative modes observed in other organisms. Kozinski (1961), employing similar experimental techniques to investigate phage T4 replication, concludes that the parental DNA of the infecting T4 phage particle contributes only a fraction of its total material to any one progeny phage particle. Kozinski suggests that the mechanism of replication of bacteriophage DNA is different from that of highly organized cells where a mitotic apparatus is present.

Using a cesium chloride density gradient it has been observed (Sueoka et al., 1959) that there is a relation between the density of deoxyribonucleic acids and their guanine-cytosine content. The relation is linear, corresponding to a change of 0.00103 in density per 1.0 per cent change in guanine-cytosine content. The Harvard workers estimate that the accuracy of their technique allows one to determine the guanine-cytosine

content of deoxyribonucleic acid from its density with a precision of about one per cent. In a simultaneous, but independent, investigation at the California Institute of Technology (Rolfe and Meselson, 1959) the results of the Harvard group were confirmed. The deoxyribonucleic acid from nine species of bacteria was analyzed to determine density and base composition. The linear relationship reported by Sueoka and his coworkers was observed. Rolfe and Meselson noted that the standard deviation of density, hence base composition, within the DNA molecular population of any one bacterial species covers less than one tenth of the range over which the mean base content varies among the nine species investigated. These workers suggest that our ideas concerning genetic coding may be altered by these results, in view of the gross difference in base composition among closely related bacteria.

Watson and Littlefield (1960), suspecting that the deoxyribonucleic acid of the Shope papilloma virus might be of the single-strand type found in bacteriophage ϕ X174 and its relative S13, employed a cesium chloride gradient in an analysis of the DNA from this animal virus. Base composition was determined by density measurement, and this value supported by two additional methods. The results thus obtained plus the molecular weight value indicate the DNA of this virus is of the double helical type.

In an investigation of the behavior of Rous sarcoma virus in rubidium chloride density gradients, Crawford (1960) found that the virus has an average buoyant density of approximately 1.18. However, the virus preparations contained particles with densities from 1.16 to 1.19. The possibility that the observed difference in density was inherited was investigated and rejected. Crawford suggests that the density heterogeneity

observed may be due to variation in the amount or composition of the outer membrane of the virus particle. In a subsequent investigation (Crawford and Crawford, 1961) a rubidium chloride gradient was used to purify Rous sarcoma virus for detailed physical analysis.

MATERIALS AND METHODS

The procedure for phage titration has now been standardized and a description is readily available (Adams, 1959).

Media

Difco nutrient broth, instead of saline, was employed for all dilution blanks in order to avoid inactivation of phage. Difco nutrient agar with 0.1 per cent added dextrose was used for all poured plates. Difco tryptose broth with thiamine plus 8.5 g Difco agar per liter was used as an overlay medium. Two media of special composition were utilized:

(1) Special tryptose broth (ST broth). At the sodium chloride concentration usually employed in growth media, T3 phages adsorb rather slowly (Watson and Maaløe, 1953). For this reason a special medium was devised for the growth of host cells which were to be lysed by phage:

Bacto-tryptose	20.0 g
NaCl	0.5 g
Dextrose	1.0 g
Thiamine hydrochloride	0.05 g
Distilled water	1000.0 ml

Adjust pH to 7.2

(2) Minimal broth. For the preparation of radioactive phosphorus (P^{32}) labeled phages the following medium was employed:

Dextrose	2.0	g
NaCl	0.5	g
KCl	3.0	g
NH ₄ Cl	1.1	g
CaCl ₂	0.11	g
MgCl ₂	0.095	g
Difco-Neopeptone	0.1	g
Tris (Hydroxymethyl) amino methane	12.1	g
Distilled water	1000.0	ml

Adjust pH to 7.2

Bacterial Cultures

The bacterial cultures used in this investigation were:

<u>Bacteria</u>	<u>Code</u>	<u>Source</u>
<u>Escherichia coli</u> B	B	U. Copenhagen, Denmark
<u>Escherichia coli</u> B/1	B/1	U. Copenhagen, Denmark
<u>Escherichia coli</u> K12 strain HfrC HfrC		U. Copenhagen, Denmark

Phage Stock Preparation

The following list describes the bacteriophages investigated, their source, and the bacterial hosts involved in their propagation.

<u>Phage</u>	<u>Source</u>	<u>Hosts</u>
T3B	Isolated by A. Eisenstark from T3 stock at U. Copenhagen, Denmark	B B/1
T3C	Isolated by A. Eisenstark from T3 stock at U. Copenhagen, Denmark	B HfrC

The appropriate bacterial host was grown at 37°C, with aeration, in ST broth to a concentration of approximately 5×10^8 viable cells/ml. An initial viable cell count was used to calibrate a photoelectric colorimeter and subsequent cell concentration estimates were based on suspension turbidity as measured in the colorimeter. At 5×10^8 viable

cells/ml the culture was infected with phage at a multiplicity of five phage per bacterium and aeration was continued until the culture lysed. The lysate was then centrifuged at $8,400 \times G$ (average force) for 40 minutes to remove unlysed bacteria and bacterial debris which might adsorb phage particles. The titer of the phage suspension at this point was about 1×10^{10} particles/ml. To concentrate a phage suspension the lysate, with debris removed, was centrifuged in the Spinco Model L centrifuge for four hours at $65,900 \times G$ (average force), the supernatant removed, and the phage pellet resuspended overnight at $5^{\circ}C$ in one ml of sterile broth. Following resuspension of the pellet the concentrated material was subjected to low speed centrifugation to remove debris, and the above cycle of high-low speed centrifugation repeated. The final pellet was resuspended in broth or $0.1M$ phosphate buffer. The phage titer after such a concentration procedure was about $1-2 \times 10^{12}$ particles/ml.

In the preparation of P^{32} labeled phage stocks the host cells were aerated in minimal medium to a viable cell concentration of about 5×10^7 cells/ml, at which time P^{32} , in the form of $PO_4^{=}$ in weak HCl, was added to a final concentration of 0.5 microcuries (μc) per ml of culture. Aeration was continued to a cell concentration of 5×10^8 and the culture was infected with phage. From this point the procedure followed was the same as that described above for the preparation of unlabeled phage stocks.

Unless otherwise indicated, T3B stocks were propagated on B/1 host cells and T3C stocks on HfrC cells. This suppressed the occurrence of T3C phage in T3B stocks.

Antisera

The antisera utilized in this study were prepared in rabbits in the Phage Lab, Kansas State University, according to the procedure described by Adams (1959).

Density Determinations

Density, in this thesis, is expressed as grams per milliliter at room temperature. Density was calculated by determining the mass of a 10 λ (λ) micropipette, filling the pipette with the unknown solution to the 10 λ calibration point, and determining the mass of the pipette and contents. The difference in mass was considered as the mass of 10 λ of the unknown solution. This value was expressed as grams per milliliter. Mass determinations were made on a Mettler semi-Micro Grammatic balance.

Reactivation of Neutralized Phage

When it was desired to reactivate phage which had been effectively neutralized by homologous antisera, the papain digestion method of Kalmanson and Bronfenbrenner (1943) was utilized. The papain solution was prepared by shaking 4 g of powdered papain (Sargent Chemical Co.) in 100 ml distilled water for two hours, followed by filtration (paper) and the addition of an equal volume of distilled water to the filtrate. The diluted filtrate was stored at 5°C. Before use, a portion of the papain solution was activated by the addition of cysteine hydrochloride to a final concentration of 1.6 per cent, and the hydrogen ion concentration adjusted to pH 7.4 with 1N NaOH. This mixture was incubated at

37°C for one hour, and was then considered as activated papain. Phage titers before and after one hour incubation at 37°C with activated papain were determined, and per cent reactivation calculated from these values. Control experiments demonstrated that activated papain did not destroy the infectivity of T3B and T3C phages, but did destroy the neutralizing capacity of their homologous antisera.

Deoxyribonucleic Acid (DNA) Extraction

An aliquot of phage stock with a titer of $1-2 \times 10^{12}$ particles/ml was shaken manually for 10 minutes with an equal volume of water-saturated phenol (Gierer and Schramm, 1956); the aqueous phase was separated by centrifugation and precipitated with two volumes of ethanol. The ethanol and aqueous layers are mixed with a glass rod and the nucleic acid "spools" on the rod (Ginoza and Guild, 1961). The precipitate was dissolved in 0.1M phosphate buffer, pH 7.2, and the process repeated until no protein appeared at the interface of the phenol-aqueous layers. The final precipitate was washed once with 70 per cent ethanol in water to minimize contamination with phenol, and dissolved in 0.1M phosphate plus 0.015M NaCl - 0.0015M trisodium citrate.

Isopycnic Gradient Centrifugation of Phage

Formation of the Density Gradient. Purified cesium chloride (CsCl), obtained from the Fairmount Chemical Company, Inc., was employed to form the density gradient. A solution of CsCl in distilled water was filtered through Whatman No. 5 filter paper and adjusted to a specific gravity of 1.510. Specific gravity values were obtained by use of a hydrometer.

To 4.5 ml of such a CsCl solution in a $\frac{1}{2}$ x 2 inch lusteroid centrifuge tube was added 0.5 ml of the desired phage suspension. The CsCl solution and phage suspension were thoroughly mixed, and the lusteroid tubes were placed in the swinging bucket rotor (SW 39) of a Spinco Model L centrifuge. The mixture was then centrifuged at 30,000 revolutions per minute at 15°C for 22 hours. During the centrifugal process a density gradient was produced in the tubes and the phage particles migrated to that position in the tube which contained a CsCl solution equal to their own density. At the end of the 22 hour centrifugal period, the rotor was allowed to coast to a stop without use of the brake.

Collection of Fractions. Two methods of fraction collection from density gradient tubes were considered feasible for routine use:

- (1) Removal of fractions from the top to the bottom of the lusteroid tubes by use of a pipette and rubber bulb.
- (2) Collection of consecutive drops by punching a hole in the bottom of the lusteroid tubes.

Preliminary trial experiments were conducted which yielded results indicating that the drop collection method was superior. As graphically illustrated in Plate I, it appears that in the drop collection method, once the band of phage was passed through the hole in the tube bottom, there is an increase in titer in subsequent fractions caused by contaminating phage from the band. These phage appear to gradually be washed out by low titer fractions passing through the hole. This results in a somewhat distorted particle distribution in the portion of the tube above the band.

When fractions are collected by pipette and bulb the distortion is reversed (Plate I). The distortion now occurs in the area of the tube below the band, apparently as a result of stirring during the pipetting

process.

It is apparent from Plate I that the band distortion during the collection process was minimum if the drop collection method was used. To standardize the drop collection method, as concerns drop size and speed, and to enable the collection process to be stopped and restarted at any time, the device illustrated in Plate II, modified from Szybalski (1960), was constructed.

Analysis of Fractions. Fractions collected from the gradients were analyzed by one, or both, of two techniques:

(1) Infectivity titration of each fraction to determine the concentration of viable phage particles. By plating varying dilutions on both B/1 and HfrC host cells, it was possible to determine the location and concentration of each type of phage.

(2) Determination of the amount of radioactivity in each fraction. Phage which are propagated in host cells grown in the presence of P^{32} incorporate P^{32} into their nucleic acid (Hershey and Chase, 1952). Thus, in a properly prepared suspension, radioactivity should come only from phage particles, and the amount of radioactivity demonstrated by any given fraction should be directly proportional to phage concentration. To analyze for radioactivity, small amounts (usually 0.05 ml) from each fraction collected were placed on copper planchets and dried under an infra-red lamp. The dried samples were placed in a Geiger-Müller chamber connected to a Model 2000 Berkeley decimal scaler, and counts per minute (CPM) values obtained.

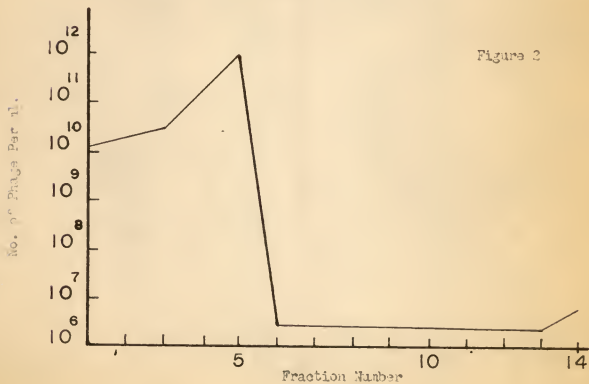
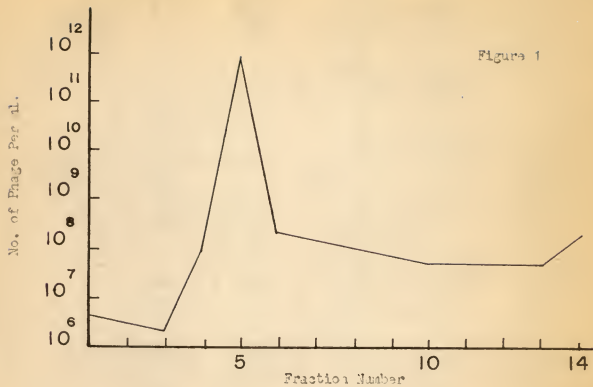
A third method of gradient analysis excluded the collection of fractions. This technique, utilizing ultraviolet optics, was designed to omit any mixing of bands or band distortion which might result from a

EXPLANATION OF PLATE I

Fig. 1. Graphic description of band distortion when fractions are collected by the consecutive drop method. Fraction 1 was originally located at the bottom of the centrifuge tube, fraction 14 at the top.

Fig. 2. Graphic description of band distortion when fractions are collected by pipette and bulb. Fraction 1 was originally located at the bottom of the centrifuge tube, fraction 14 at the top.

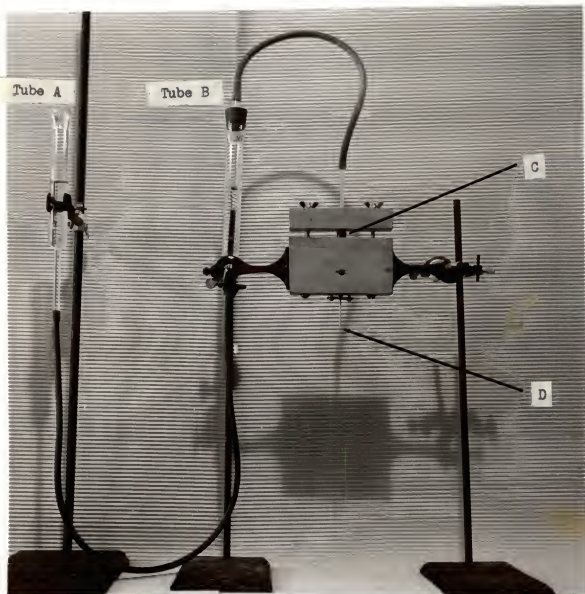
PLATE I



EXPLANATION OF PLATE II

With the water in tubes A and B at the same level, the rubber seal C is placed at the top of the centrifuge tube contained in the wooden block. When the wing nuts are tightened, the rubber seal C pushes the centrifuge tube downward until it is pierced by the needle D, which is embeded in a second rubber seal. By increasing the height of tube A, the water level in tube B is raised, causing a positive pressure in the centrifuge tube and drops can be collected from the tip of needle D. By decreasing the height of tube A, the water level in tube B is lowered, causing a negative pressure in the centrifuge tube. This allows curtailment of the drop collection process.

PLATE II



fraction collection method. The CsCl-phage mixtures were centrifuged in quartz tubes designed for use in the SW 39 rotor. At the end of the centrifugation process the quartz tubes were positioned in a quartz tube scanner, a device which replaces the usual cell compartment on the Beckman DU spectrophotometer, and optical density (OD) determinations were obtained at one millimeter increments throughout the length of the tubes. By measuring OD at 270 millimicrons ($m\mu$) it was possible to determine the positions of phage particles in the gradient. This method appeared to offer no advantage over the drop collection method, and was not used routinely since fractions were not available for further analysis.

Gradient Centrifugation of Nucleic Acid

Isopycnic Gradient Centrifugation. The procedure for isopycnic gradient centrifugation of DNA is the same as that described for phage, with the exception of minor technical changes. One exception concerns the density of the initial CsCl solution. Since DNA molecules are considerably more dense than intact phage particles, a more dense initial CsCl solution is required in order to form a gradient which includes the density of DNA. This increase in density requires either a decrease in the volume contained within the centrifuge tube, or a reduction in the speed at which the rotor is operated. In these experiments, in order to minimize the amount of time required for DNA to reach an isopycnic position, the volume contained within the centrifuge tube was decreased.

To 3.0 ml of 9.3 molar CsCl in lusteroid tubes was added 0.3 ml of DNA solution. After gentle mixing this mixture was overlaid with 1.2 ml cottonseed oil. The lusteroid tubes were then centrifuged in the SW 39 rotor at 35,000 r.p.m. for 75 hours. At the end of the centrifugation

process, fractions were collected by the drop collecting device described in Plate II. Enough diluent was added to each fraction to result in a final volume of 1.7 ml, and optical density readings were made at 260 $m\mu$ in a Beckman model DU spectrophotometer.

Gradient Differential Centrifugation. To determine if a difference in sedimentation rate exists between DNA extracted from T3B and T3C a sucrose gradient was constructed which at its maximum density was less dense than DNA molecules. Thus, if subjected to a long enough period of centrifugation, the molecules would migrate to the bottom of the tube. However, if the centrifugation process is curtailed before the molecules have migrated to the bottom of the tube, the sucrose gradient will prevent convective disturbances and the molecules can be observed. Molecules varying in size, shape, or density sediment at different rates and can be observed at different positions within the gradient.

To construct a preformed sucrose gradient equal amounts of sucrose solutions of decreasing density 1.340, 1.242, 1.144, and 1.095 were layered in quartz tubes with a $\frac{1}{2}$ ml syringe and 2 inch 22 gauge needle. These tubes were allowed to stand 24 hours to allow the interfaces to diffuse and form a more continuous gradient. The DNA solution was gently placed over the sucrose and centrifuged at 36,000 r.p.m. for desired time intervals. Drops were collected by punching a hole in the bottom of the tube. The contents of each fraction were diluted to a final volume of 1.7 ml, and optical density readings were made at 260 $m\mu$ in a Beckman DU spectrophotometer.

RESULTS

Density Relationship of T3B and T3C

Preliminary experiments indicated that T3B and T3C might differ in density (Eisenstark and Van Sickle, 1961). Two complications were present in these early density gradient studies:

- (1) Less than 10 per cent of the total phage assayed as plaque-forming units after being subjected to density gradient centrifugation.
- (2) A second peak titer of each phage type was present at the top of the gradient.

In the present investigation, two methods were devised to solve the inactivation problem involved in the early studies. To follow the behavior in a density gradient of the phages which were inactivated, phage were labeled with P^{32} and the amount of radioactivity in each fraction collected was determined. The results illustrated in Plate III indicate that there is an absolute relationship between the radioactivity peak and the peak of infectivity. Thus one may assume that the inactivated phages band at the same position in the gradient as the viable phage which can be located by infectivity titrations.

A second method of eliminating the inactivation problem was to replace the 98 per cent pure CsCl employed in early studies with purified CsCl. As shown in Table 1, no inactivation occurred when T3B or T3C were subjected to density gradient centrifugation involving purified CsCl, whereas if 98 per cent pure CsCl was used, the amount of inactivation was great.

The use of purified CsCl also helped mitigate the second discrepancy encountered in the early studies, but the second peak was never entirely

EXPLANATION OF PLATE III

Diagram showing the relationship between radioactivity peak and infectivity peak. Legend: Line (O) = T3B infectivity titer
Line (X) = counts per minute (CPM)

Phage Employed

Initial infectivity titer.	CPM of 0.05 ml of initial phage suspension.
T3B 1.8×10^{11}	2,700

Composition of Gradient

T3B	0.5 ml
CsCl (Sp. Gr. 1.510)	4.5 ml

Details of Centrifugation

Rotor	SW 39
Speed	30,000 r.p.m.
Time	22 hours

Method of Analysis

Fractions were collected by punching a hole in the bottom of the tube. Fraction 1 was contained at the bottom of the gradient tube, fraction 17 at the top.

Infectivity titer was analyzed by making serial dilutions of each fraction and plating the dilutions on E. coli B/1.

Radioactivity determinations were made by drying 0.05 ml samples from each fraction on copper planchets. Each sample was counted for two minutes.

PLATE III

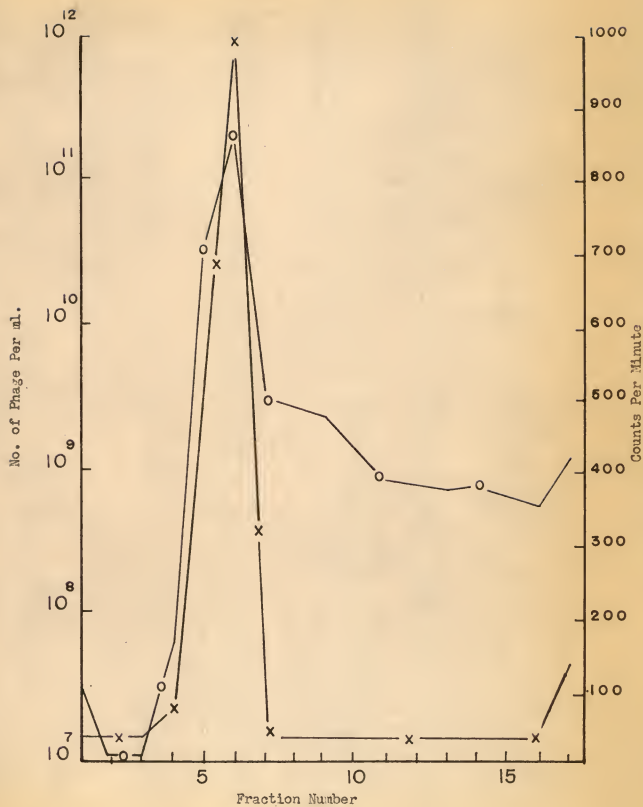


Table 1. Comparison of phage inactivation by 98 per cent pure CsCl and purified CsCl. Phage were centrifuged in CsCl at $71,440$ (av) \times G for 22 hours, resuspended, and titered.

CsCl employed :	Phage :	Initial titer :	Titer after : centrifugation :	Per cent inactivation :
98% Pure	T3B	2.3×10^7	8.4×10^4	99.7
	T3C	4.4×10^6	1.0×10^4	99.8
Purified	T3B	2.3×10^7	2.5×10^7	none
	T3C	4.4×10^6	4.4×10^6	none

eliminated and this matter will be discussed later in this thesis.

Utilizing the methods of band detection described, experiments were designed to determine the particle density of T3B and T3C. In an initial experimental series an attempt was made to demonstrate a particle density difference between T3B and T3C using radioactivity for band detection. One centrifuge tube contained P^{32} labeled T3B only, a second tube labeled T3C only, and the final tube contained T3B and T3C both labeled. At the end of centrifugation the contents of all three centrifuge tubes were divided into 30 equal fractions by drop collection. The amount of radioactivity in each fraction was determined. The density of the contents of the fraction which contained the radioactivity peak was also determined. Table 2 summarizes the results of a typical experiment in this series.

It was evident that if a density difference actually exists, a more refined technique must be employed for its detection. The only information from Table 2 which might suggest a density difference is the fact that the band width was increased when the two particles were centrifuged in the same CsCl gradient tube. Phage concentrations in the three tubes were equal, so the observed increase in band width was not due to concentration variation.

Table 2. Density of phages T3B and T3C determined by use of P^{32} labeled phages. 0.5 ml of phage, 5×10^{11} particles/ml, was added to 4.5 ml CsCl (Sp. Gr. 1.510).

Contents of centrifuge tube	No. of fractions collected	Fraction containing most radioactivity	Density of fraction containing most radioactivity	No. of fractions in entire peak
T3B	30	7	1.49	3
T3C	30	7	1.49	3
T3B and T3C	30	7	1.49	4

To further investigate the density relationship, experiments were devised in which both radioactivity and infectivity of the various fractions were determined. P^{32} labeled T3B and T3C in equal concentration, 2×10^{11} particles/ml, were centrifuged in the same centrifuge tube in CsCl. At the end of 22 hours of centrifugation fractions were collected as drops through a hole in the bottom of the tube. To maintain a sufficient volume in each fraction to allow analysis of both radioactivity and infectivity, four drops per fraction were collected. For radioactivity analysis 0.05 ml from each fraction was dried on copper planchets and CPM determined. The remainder of each fraction was serially diluted, and the various dilutions were plated on B/1 and HfrC to determine the titer of T3B and T3C in each fraction. In two such analyses the peak titer of T3B and T3C occurred in the same fraction, and the data obtained from radioactivity measurements did not indicate the presence of two bands. In a third analysis, graphically presented in Plate IV and Plate V, again only one radioactivity peak was detected. However, in this analysis the peak titers of T3B and T3C occurred in separate consecutive fractions. A repeated titration from the original 10^3 dilution tubes gave the same results.

EXPLANATION OF PLATE IV

Radioactivity analysis of isopycnic density gradient centrifugation of phages T3B and T3C. The infectivity analysis of this tube appears in Plate V.

Phage Employed

	Initial infectivity titer	CPM of 0.05 ml of initial phage suspension
T3B	2×10^{11}	1,240
T3C	2×10^{11}	1,400

Composition of Gradient

T3B	0.25 ml
T3C	0.25 ml
CsCl (Sp. Gr. 1.510)	4.5 ml

Details of Centrifugation

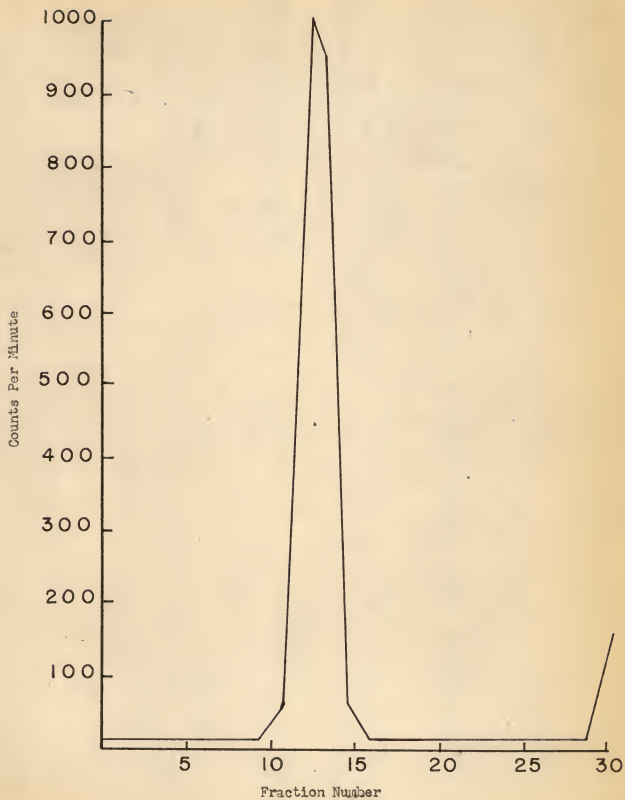
Rotor	SW 39
Speed	30,000 r.p.m.
Time	22 hours

Method of Analysis

The gradient was collected, in fractions of four drops each, by punching a hole in the bottom of the centrifuge tube.

0.05 ml samples from each fraction were dried on copper planchets and CPM of each fraction determined. Each sample was subjected to a two minute counting period.

PLATE IV



EXPLANATION OF PLATE V

Infectivity analysis of isopycnic density gradient centrifugation of phages T3B and T3C. The radioactivity analysis of this tube appears in Plate IV.

Legend: Line (O) = T3B infectivity titer
Line (X) = T3C infectivity titer

Phage Employed

Initial infectivity titer.	Assay Host
T3B 2×10^{11}	B/1
T3C 2×10^{11}	HfrC

Composition of Gradient

T3B	0.25 ml
T3C	0.25 ml
CsCl (Sp. Gr. 1.510)	4.5 ml

Details of Centrifugation

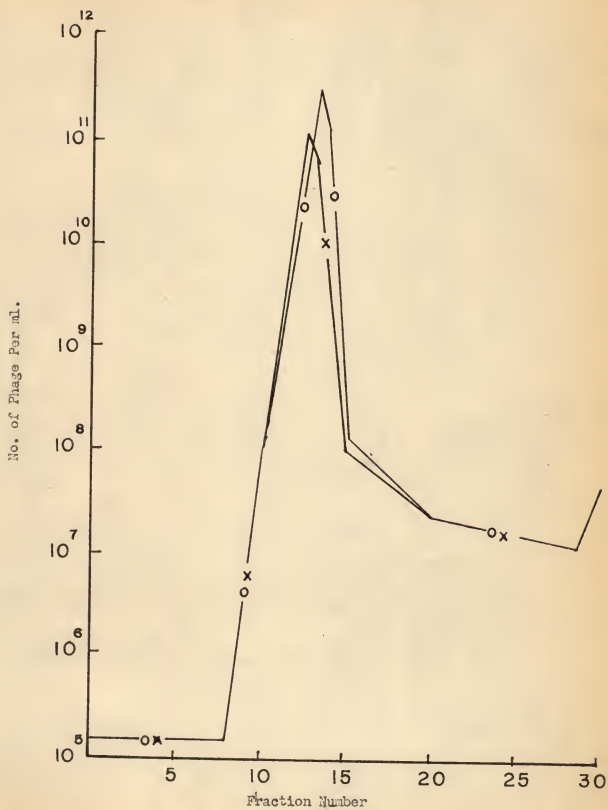
Rotor	SW 39
Speed	30,000 r.p.m.
Time	22 hours

Method of Analysis

The gradient was collected, in fractions of four drops each, by punching a hole in the bottom of the centrifuge tube.

Serial dilutions of each fraction were plated on *E. coli* B/1 and HfrC to determine the number/ml of each phage type.

PLATE V



Also to be observed in Plate V is the fact that the plot of T3C approaches the peak area earlier than the plot of T3B, and declines from the peak area before the plot of T3B. This fact, in conjunction with the observation that the peak titer of T3C occurs in a more dense fraction than the peak titer of T3B, might indicate a very slight (less than four drops) mean density difference between these two phage types.

Because the infectivity analyses furnished more precise data, and indicate that there is a slight density difference between the two phages, it appeared worthwhile to perform an experiment in which single drops were analyzed for infectivity. Since the removal of fractions from the top of the gradient by pipette results in band distortion, the collection of single drops passing through a hole in the bottom of the tube appeared to be the most reliable way to finely fractionate the contents of a centrifuge tube. In these experiments a method was employed which made it unnecessary to analyze every drop. The three tubes in the SW 39 rotor were prepared in an identical manner, so that at the end of centrifugation the phage bands in all three tubes should be in the same position. The contents of the three tubes, following centrifugation, were collected in this manner:

- Tube 1. Six drops were collected in each fraction.
- Tube 2. One drop was collected in each fraction. About 120-126 fractions usually resulted from this method of collection.
- Tube 3. This tube was a spare tube. Collections were made from it only if a failure occurred during centrifugation or in the collection process of the previous two tubes.

The fractions collected from tube one were diluted and a 10^9 dilution plated on E. coli B, the host which can be infected by both phage strains. At such a high dilution only the fractions which contain the phage band

will produce plaques. Thus, by multiplying the number of the fraction producing the greatest number of plaques by six (the number of drops per fraction) one can estimate the range of single drops from tube two which will contain the phage band. Application of this method necessitates the titration of only 20 drops instead of the entire series of 120-130 drops.

Plate VI illustrates the results obtained in the first of such experiments. The results of two identical confirmatory experiments were essentially the same; the peak of T3C infectivity appeared one drop earlier than the peak of T3B infectivity. A second fact, observed in all three experiments and apparent in Plate VI, is that although the plots of T3C and T3B concentration are separated before the peaks, as one would expect if T3C is indeed more dense, they decrease after the peak is past almost as a single line.

Investigation of Density Variants

It is evident in the plates thus far presented that all phages are not contained within the concentrated band. Phage are always detectable throughout the gradient, in concentrations dependent upon the concentration of phage added to the gradient. Plate III graphically illustrates phage T3B concentration from the bottom of the centrifuge tube to the top. It should be noted from Plate III that increases in phage concentration occur both at the bottom and top of the centrifuge tube. The increase in titer in the portion of the tube above the peak has been previously discussed (Materials and Methods).

To decide whether the phage present outside the band are genotypic density mutants, or phenotypic density mutants resulting from errors in

EXPLANATION OF PLATE VI

Infectivity analysis of single drops collected after isopycnic density gradient centrifugation of phages T3B and T3C.

Legend: Line (O) = T3B infectivity titer
Line (X) = T3C infectivity titer

Phage Employed

	Initial infectivity titer.	Assay Host
T3B	1 x 10 ¹⁰	B/1
T3C	1 x 10 ¹⁰	HfrC

Composition of Gradient

T3B	0.25 ml
T3C	0.25 ml
CsCl (Sp. Gr. 1.510)	4.5 ml

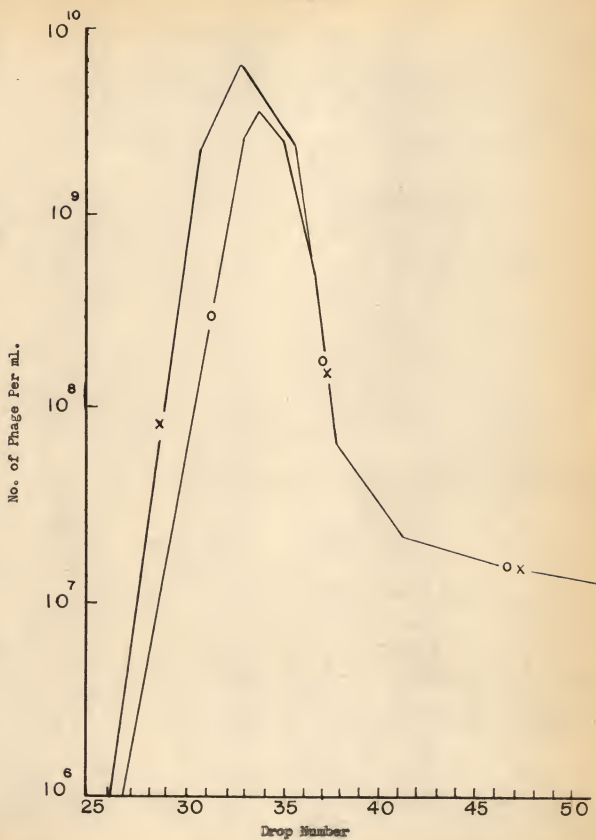
Details of Centrifugation

Rotor	SW 39
Speed	30,000 r.p.m.
Time	22 hours

Method of Analysis

Drops were collected by a hole in the bottom of the centrifuge tube. Serial dilutions of each drop were plated on E. coli B/1 and HfrC to determine the number/ml of each type of phage.

PLATE VI



assembly of phage particles, experiments were performed as described below. Phage T3B was banded in a CsCl gradient. Material from three locations in this gradient, the top, the band, and the bottom, was centrifuged in separate tubes in CsCl and the resulting gradients analyzed to determine band position. Plate VII portrays the original T3B distribution in a CsCl gradient. Plate VIII illustrates the band position of the material centrifuged from the three locations, marked by an X, in the original distribution shown in Plate VII.

Material from each of the three fractions discussed above was used to infect and lyse E. coli B/1 cells, and the resulting progeny phage centrifuged in CsCl. The progeny from all three fractions formed concentration distributions the same as that of the original T3B phage, as illustrated in Plate VII.

Nucleic Acid Studies

Due to the regularity of mutation from T3B to T3C it is of interest to examine the genetic material of these two phage types in hopes of obtaining data which would suggest a mechanism for the mutation.

Although there are reports in the literature of taxonomically closely related species varying widely in the density of their nucleic acid content (Rolfe and Meselson, 1959), it seemed unlikely that a detectable density change would be observed in the present case. However, before sedimentation studies can be interpreted, density relationship must be determined. To this end CsCl density gradients were constructed which would band nucleic acids extracted from phages T3B and T3C. As illustrated in Plate IX, the nucleic acid from both phages bands at the same position in the gradient.

EXPLANATION OF PLATE VII

Concentration distribution of a stock suspension of phage T3B. Fractions were collected by punching a hole in the bottom of the centrifuge tube. Material from the fractions marked with an X was respun in CsCl, the concentration distributions which resulted appear in Plate VIII.

Phage Employed

Initial infectivity titer.	Assay Host
T3B 1×10^{11}	B/1

Composition of Gradient

T3B	0.5 ml
CsCl (Sp. Gr. 1.510)	4.5 ml

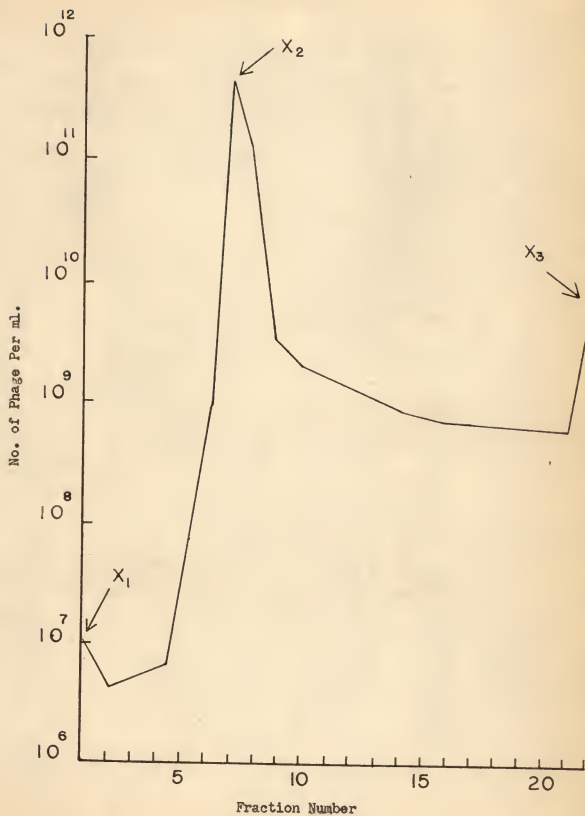
Details of Centrifugation

Rotor	SW 39
Speed	30,000 r.p.m.
Time	22 hours

Method of Analysis

Fractions were collected by a hole in the bottom of the centrifuge tube. Serial dilutions of each fraction were plated on B/1 to determine T3B titer.

PLATE VII



EXPLANATION OF PLATE VIII

Concentration distributions, in CsCl density gradients, of phages taken from three different fractions of the same gradient. The original gradient is illustrated in Plate VII.

Legend: Line (X_1) = material from fraction 1, Plate VII
Line (X_2) = material from fraction 7, Plate VII
Line (X_3) = material from fraction 22, Plate VII

Phage Employed

Initial infectivity titer.	Assay Host
T3B Variable, depending on the fraction used.	B/1

Composition of Gradient

T3B from indicated fraction of the previous gradient	0.2 ml
Distilled H ₂ O	0.4 ml
CsCl (Sp. Gr. 1.510)	4.5 ml

The distilled water was added to replace the amount of water usually added as part of the phage suspension. This kept the gradients in Plate VII and Plate VIII comparable.

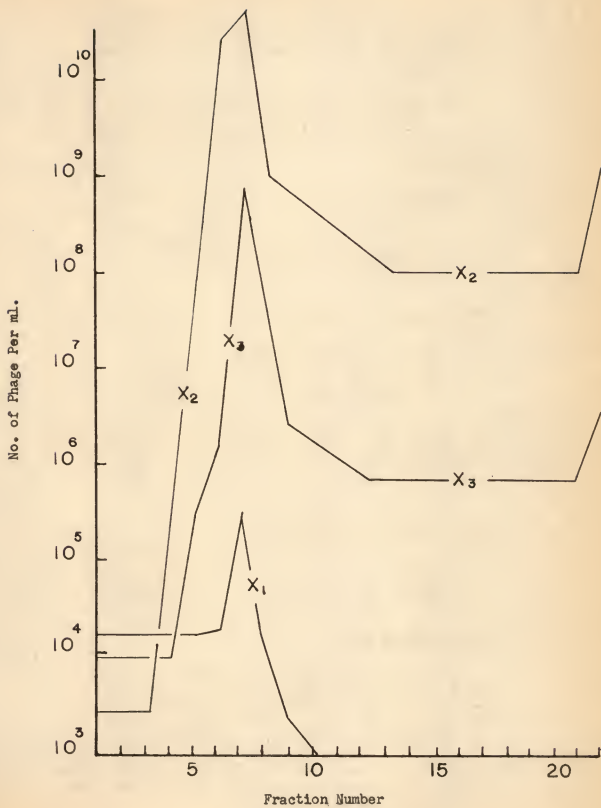
Details of Centrifugation

Rotor	SW 39
Speed	30,000 r.p.m.
Time	22 hours

Method of Analysis

The contents of the three gradient tubes were collected as drops through a hole in the bottom of the centrifuge tube. Twenty-two fractions were collected from each tube, and the number of phage particles/ml in each fraction determined. Although three separate gradients were involved, since the composition of the gradients and number of fractions collected are equal, phage concentrations in the three tubes were plotted on a common graph.

PLATE VIII



EXPLANATION OF PLATE IX

Spectrophotometric analysis of isopycnic density gradient centrifugation of DNA extracted from phages T3B and T3C.

DNA Employed

DNA solutions from the two phages were adjusted to equal concentrations. A 1:1 mixture of the nucleic acids was used in the gradient described by this plate.

Composition of Gradient

DNA solution (T3B and T3C, 1:1)	0.3 ml
CsCl (9.3 molal) mixed well	3.0 ml
Cottonseed oil	1.2 ml

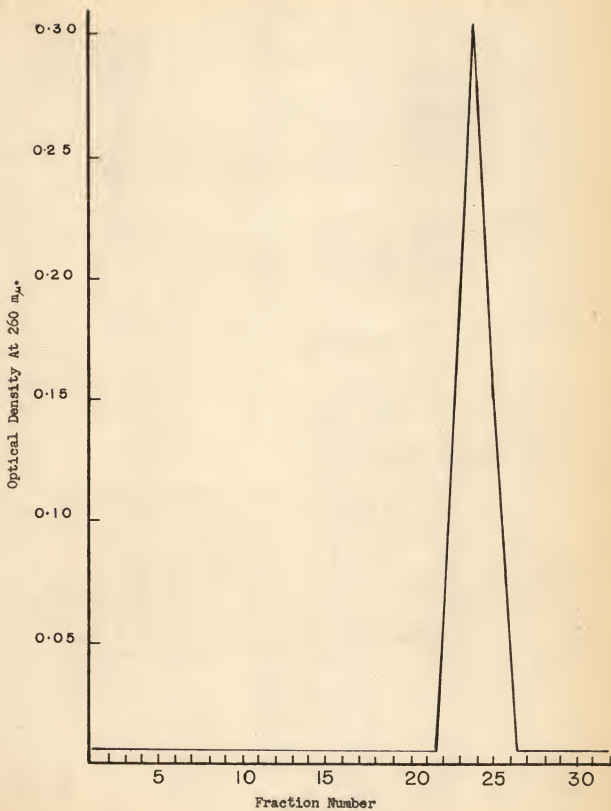
Details of Centrifugation

Rotor	SW 39
Speed	35,000 r.p.m.
Time	75 hours

Method of Analysis

At the end of centrifugation fractions were collected, four drops per fraction, by a hole in the bottom of the tube. Enough diluent was added to each fraction to result in a final volume of 1.7 ml, and optical density readings were made at 260 m μ in a Beckman DU spectrophotometer. One gradient tube contained distilled water in place of DNA solution. This tube was divided into fractions, the fractions diluted to 1.7 ml and used as a blank for spectrophotometric readings instead of distilled water. This would account for any difference in OD resulting from increasing CsCl concentration from top to bottom of the gradient.

PLATE IX



Assuming identity in molecular shape and in density, any difference in sedimentation rate in a common medium would be due to variation in size. A solution of nucleic acid extracted from T3B and T3C was placed over a preformed sucrose gradient and centrifuged for 13 hours. The maximum density of the gradient was considerably less than the density of the nucleic acids involved; hence if centrifugation had been continued for a long enough period of time, the molecules would have been sedimented to the bottom of the tube. Since centrifugation was curtailed before the migrating molecules reached the tube bottom, the nucleic acid boundary(s) was stabilized by the sucrose gradient in a position within the tube. Plate X illustrates the results of such a centrifugation.

Effect of Antibodies on Phage Particle Density

During the course of this investigation an interesting observation was made concerning the phage-antibody complex. Prior experiments reported in this thesis indicated that if a density difference exists between T3B and T3C it is very small. The phages were observed to band in the same vicinity of the gradient tube. The possibility was considered that antibodies, being protein and thus less dense than the intact phage particles, could be employed to cause the phages to band at different positions within the gradient. One might expect that, if the antigen (phage)-antibody union could withstand the opposing forces exerted upon its parts during the formation of the gradient, the protein antibodies attached to the phage particle would lower its density.

To investigate this possibility a gradient was constructed of the following materials: T3C, T3B, and anti-T3B, in CsCl. Since the inclusion

EXPLANATION OF PLATE X

Spectrophotometric analysis of gradient differential centrifugation of DNA extracted from phages T3B and T3C.

DNA Employed

DNA solutions from the two phages were adjusted to equal concentrations. A 1:1 mixture of the nucleic acids was used in the analysis described in this Plate.

Composition of Gradient

A sucrose gradient was formed by layering 1.0 ml amounts of sucrose solutions of decreasing density: 1.340, 1.242, 1.144 and 1.095. The layers were allowed to diffuse for 24 hours to smooth the interfaces. The DNA solution, 1.0 ml, was carefully layered on top of the gradient and the centrifuge started.

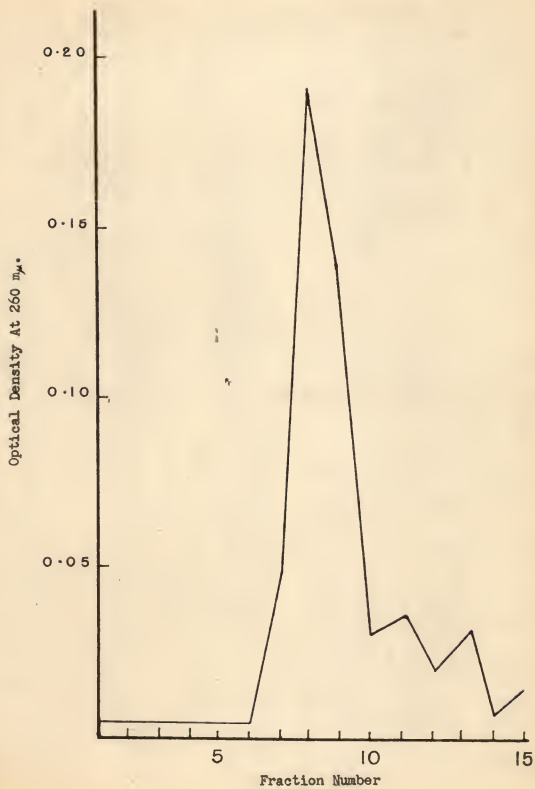
Details of Centrifugation

Rotor	SW 39
Speed	36,000 r.p.m.
Time	13 hours

Method of Analysis

At the end of centrifugation fractions were collected by drops. Enough diluent was added to each fraction to give a final volume of 1.7 ml and optical density readings were made at 260 m μ in a Beckman DU spectrophotometer. One gradient tube contained distilled water in place of DNA solution. This tube was divided into fractions, the fractions diluted to 1.7 ml, and used as a blank for spectrophotometric readings instead of distilled water. This would account for any difference in OD resulting from the increasing sucrose concentration from top to bottom of the gradient.

PLATE X



EXPLANATION OF PLATE XI

Radioactivity analysis of isopycnic density gradient centrifugation of a mixture of phages T3B, T3C, and rabbit anti-T3B serum.

Phage Employed

Initial infectivity titer.	CPM of 0.05 ml of initial phage suspension
T3B 2×10^{11}	1,240
T3C 2×10^{11}	1,400

Composition of Gradient

T3B, T3C and anti-T3B serum (1-10 dilution in saline) were mixed in equal quantities and incubated at 37°C for 45 minutes. This was called mixture A.

Mixture A	0.5 ml
CsCl (Sp. Gr. 1.510)	4.5 ml

Details of Centrifugation

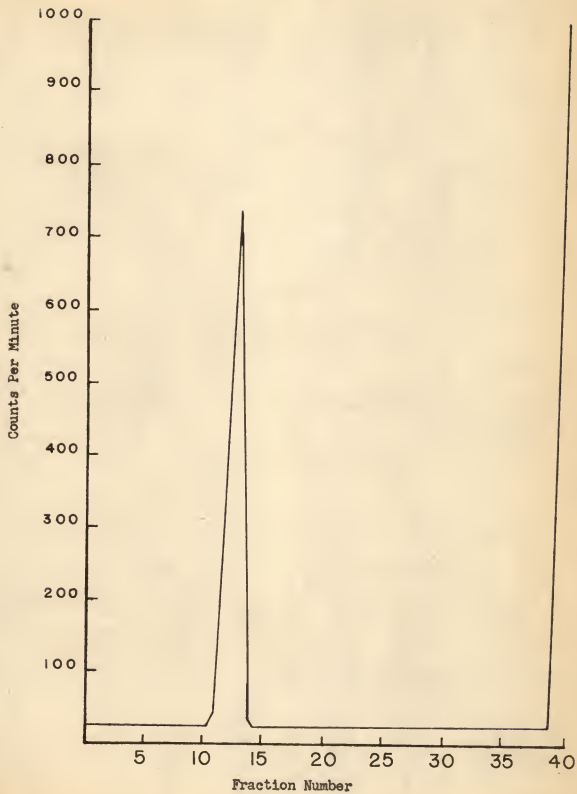
Rotor	SW 39
Speed	30,000 r.p.m.
Time	22 hours

Method of Analysis

The gradient was collected, by punching a hole in the bottom of the tube, into 40 fractions.

0.05 ml samples from each fraction were dried on copper planchets and CPM of each fraction determined. Each sample was subjected to a two minute counting period.

PLATE XI



of anti-T3B results in inactivation of T3B, radioactively (P^{32}) labeled phages were employed. An analysis of such a gradient is illustrated in Plate XI. One should contrast these results with the results of a similar experiment in which no antiserum was present (Plate IV). Although a peak occurs in both cases at the extreme top of the centrifuge tube, the magnitudes of the peaks vary greatly. It was determined by plating dilutions of the fractions on E. coli HfrC that the T3C infectivity peak occurs in fraction 12 with one of the radioactivity peaks. The T3C titer at the second radioactivity peak, fraction 40, was very low. Due to the action of the antiserum, infectivity of T3B could not be determined.

In an attempt to further show that the radioactivity peak in fraction 40 was due to the presence of P^{32} labeled T3B, altered in density by antibody, an attempt was made to reactivate neutralized phage and perform infectivity titrations for T3B. A CsCl gradient was formed containing unlabeled T3B, unlabeled T3C, and anti-T3B serum. Fractions were collected and analyzed in the following manner:

(1) 0.1 ml from each fraction was diluted into 9.9 ml of nutrient broth. From these dilution tubes a 10^3 dilution of each fraction was plated on B/1 and a 10^3 , 10^5 and 10^7 dilution on HfrC.

(2) 0.1 ml from each fraction was diluted into 9.9 ml of activated papain and incubated at 37°C for one hour. Following incubation a 10^3 dilution of each fraction was plated on B/1 and a 10^3 , 10^5 and 10^7 dilution on HfrC.

The results of these experiments are graphically summarized in Plate XII. The activated papain did not change the T3C titer. The change in T3B titer effected by the papain is illustrated.

EXPLANATION OF PLATE XII

Infectivity analysis of isopycnic density gradient centrifugation of a mixture of phages T3B, T3C and rabbit anti-T3B serum. Titters before and after incubation with activated papain are shown.

Legend: Line (X_1) = T3C titer before and after treatment with activated papain.
Line (X_2) = T3B titer after treatment with activated papain.
Line (X_3) = T3B titer before treatment with activated papain.

Phage Employed

Initial infectivity titer.	Assay Host
T3B 1.0×10^{11}	B/1
T3C 1.1×10^{11}	HfrC

Composition of Gradient

T3B, T3C and anti-T3B serum (1-10 dilution in saline) were mixed in equal quantities and incubated at 37°C for 45 minutes. This was called mixture B.

Mixture B	0.5 ml
CsCl (Sp. Gr. 1.510)	4.5 ml

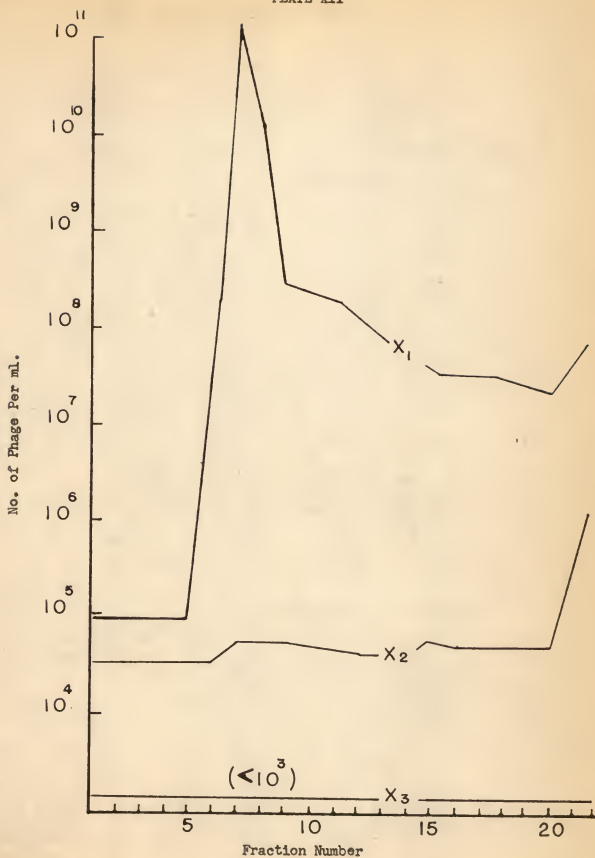
Details of Centrifugation

Rotor	SW 39
Speed	30,000 r.p.m.
Time	22 hours

Method of Analysis

The gradient was collected by punching a hole in the bottom of the tube. 0.1 ml of each fraction was diluted into 9.9 ml of nutrient broth and various dilutions were plated on B/1 and HfrC. 0.1 ml of each fraction was diluted into 9.9 ml activated papain and incubated at 37°C for one hour. Following incubation various dilutions of the fractions were plated on B/1 and HfrC.

PLATE XII



In similar experiments involving labeled T3B, labeled T3C and anti-T3C obtained from early bleedings of T3C injected rabbits these results are obtained: the T3B can be located within the gradient at one radioactivity peak by infectivity analysis, the second radioactivity peak contains very few T3B particles and is apparently caused by labeled inactivated T3C.

DISCUSSION

The dissident results obtained in one series of identical experiments deserve comment. It has been stated that the results depicted in Plate V were obtained in only one of three identical analyses, and it might appear in error to describe the information obtained in this exceptional experiment at the expense of the two experiments whose results agree. However, if one considers the manner in which drops are collected into fractions, this exceptional experiment becomes very significant.

Assume that T3B and T3C peak titers are separated by one drop in a gradient, and that the gradient is collected as four drops per fraction. In the sequence below let drop 33 contain the T3B peak titer and drop 34 the T3C peak titer.

28-29-30-31-32-33-34-35-36-37-38-39

It is evident that if drops 28-31 were collected as one fraction, and drops 32-35 as a second fraction, then an analysis of fractions would indicate that the T3B and T3C peak titers were contained in the same fraction. In contrast, if drops 30-33 were collected as a fraction, and drops 34-37 as a fraction, then an analysis of fractions would indicate that the T3B and T3C peak titers were contained in separate consecutive

fractions. Such may have been the case in the series of three experiments considered; such an interpretation is supported by the experiments in which single drops were analyzed.

A second point of interest concerns the separation of plots of T3B and T3C infectivity as the band is approached, and the union of these plots when the band is passed. Such behavior is evident in Plate V and Plate VI. The single drop analyses suggest that T3C is slightly more dense than T3B; thus it is not surprising to observe the T3C plot lead the plot of T3B as the band is approached; however, by the same reasoning one would assume that the plot of T3C would decline ahead of the T3B plot after the peak is past. Such is not the case. Several different possibilities might account for this deviation from the expected.

(1) The first T3C particles pass freely out of the hole in the tube, but the T3C particles in an area of greater T3B concentration are held back, possibly due to their possession of a longer tail appendage. This would result in a one-sided widening of the T3C band.

(2) There is a greater density heterogeneity among the T3C particles, resulting in a uniformly wider T3C band.

(3) Although the initial concentrations are equal, more T3B toxicity might occur, and by using infectivity to detect phage a misleadingly narrow T3B band would occur. In view of the results presented in Table 1 and Table 2, this idea seems very unlikely. Although the problem is not treated further in this work, the results illustrated in Plate VI seem to favor the second proposal.

Attention should be directed to the increase in infectivity titer which is observed at the top and bottom of the gradient tube (Plate III).

In view of the results obtained by Crawford (1960) concerning phenotypic density heterogeneity, these increases in titer might be explained as follows: in the assembly of the phage particles variations occur which result in the formation of a wide range of phenotypic density variants. These variants are responsible for the low titer which is always detectable throughout the gradient tube. Such variants would be confined within the limits of the gradient: all density variants more dense than the maximum density of the gradient would accumulate at the bottom of the tube, all variants less dense than the least dense region of the gradient would accumulate at the top of the tube. This would account for the increases in titer at the bottom and top of the gradient. The results of experiments described graphically in Plate VII and Plate VIII show that the present system is not analogous to the system investigated by Crawford, and the above interpretation is not valid. The present results indicate that not only is the density variation not genotypic, but that it is not even phenotypic (Plate VIII). Were the differences in density truly phenotypic, the material from fraction 1 would return to that fraction in a new gradient, as would the material from fraction 22 return to fraction 22. The fact that material from all fractions, upon recentrifugation in a gradient, forms a peak at the same location as the original stock suspension would suggest that:

(1) The phage located outside the band, in the original gradient, are "unclean", possibly having cellular debris attached to alter their density. Most of this cellular debris would be removed in the second centrifugation, resulting in a majority of the population returning to their genetically determined density of 1.49 g/ml.

(2) The shaking involved in the deceleration process results in the distribution of a few phage throughout the gradient. Although the data presented seem to fit the first suggestion quite well, the second possibility cannot be ruled out on the basis of information presented here.

The nucleic acid studies presented are admittedly incomplete. From the few results obtained it is believed that if differences in density or size of the genetic material exist, they are very subtle and should be examined by techniques more refined than the ones available for this study. Sedimentation, in a uniform aqueous medium, followed with ultraviolet optics during the actual centrifugal process would be the choice method of study. Differences in sedimentation rate of DNA from T3B and T3C would be indicative of a loss or gain of genetic material during the mutational process. In the absence of an analytical centrifuge, the procedure employed in this study could be refined by the use of a more concentrated DNA solution. This would allow the collection of smaller fractions, even single drops. Micro-cuvettes could also be handily employed.

Several interesting facets are disclosed by the observations on the behavior of the phage-antibody complex in a density gradient. The data derived from experiments utilizing P^{32} labeled phages are very convincing (Plate XI). However, since a small CPM peak occurs at the top of the gradient in the absence of homologous antiserum (Plate IV), a final conclusion concerning the location of the inactivated T3B is prohibited. A comparison of the magnitudes of the two peaks strongly favors the hypothesis that the radioactivity peak at the top of the gradient is due to the presence of inactivated labeled T3B. The small radioactivity peak

observed at the top of every gradient involving P^{32} labeled phages is probably best explained by the observation of Maaløe and Stent (in Watson and Maaløe, 1953) that during the last 10 minutes before normal lysis, large amounts of phosphorus-containing phage material is produced in the cells which is not developed into mature phage before lysis. The phage purification process employed in this study might not separate all of this phosphorus-containing material from the functional phage particles. This material might account for the small radioactivity peak at the top of each gradient. The possibility of the presence of phospholipids in cellular debris should also be considered. It is apparent that the increase in infectivity titer at the top of the gradient is not large enough to account for the observed increase in radioactivity at this point. In Plate III, for example, the infectivity titer in fraction 17 would have to be 7.5×10^{10} to account for the radioactivity measured in that fraction. This figure is calculated from the relation of radioactivity to infectivity titer at the peaks.

The reactivation, by activated papain, of the neutralized T3B particles is unimpressive. However, the maximum amount of reactivation does occur in the fraction which, from the radioactivity data, is thought to contain the neutralized T3B. Phage particles which are just neutralized can be readily reactivated by papain, while over-neutralized phage are not reactivated (Kalmonson and Bronfenbrenner, 1943). The difficulty encountered in this study was that in order to combine enough antibody to the phage to lower its density enough that it bands at the top of the gradient, the phage must be neutralized beyond the degree reactivable by activated papain digestion.

Since it is obvious from the data presented that the phage-antibody

complex is indeed located at the top of the gradient after centrifugation, some interesting ideas concerning the antigenic composition of these phage particles can be formulated. The antigenic make-up of these two phages has previously been pictured as follows (Eisenstark, Maaløe, and Birch-Andersen, 1961):

	T3B	T3C
Neutralizable antigen	b	b, c
Head antigen	X	X

The b antigen on T3C is functional in stimulating anti-b antibodies, but the union of anti-b antibodies with antigen b does not result in the inactivation of T3C. This is presumably due to the position of the b antigen on T3C. From this antigenic picture, one would expect anti-T3B to produce a comparable density change on both types of phage, since the b antigen is present on both phage types and the head antigens are considered the same. The results (Plate XI and Plate XII) clearly do not support this idea. To explain the results obtained these suggestions are made:

- (1) There is a greater amount of b antigen on T3B than on T3C, resulting in the binding of larger quantities of anti-b protein to the T3B phages.
- (2) The b antigen on T3C is concealed in some manner which makes it less readily combinable with anti-b protein.
- (3) There are qualitative antigenic differences in the two phages which have not been previously considered, probably involving the head antigens.

The first two possibilities are considered in the light of information reported by Lindell (1961). This investigator observed that when T3C was injected into rabbits, the serum from early bleedings would neutralize

only T3C, but the serum obtained from later bleedings would neutralize T3B and T3C.

The third suggestion appears much more likely. Considering the large amount of head protein in a phage particle, reportedly antigenic in other phages (Lanni and Lanni, 1953), it would seem probable that density alterations would be produced by the combination of antibodies against this head protein. This should result in a density alteration of a phage in the presence of heterologous antiserum (ex. T3C phage and T3B antiserum), assuming the head proteins are the same. Such results are not obtained. The results which are obtained seem best explained by postulating a qualitative difference in the head antigens of T3B and T3C. Future experiments, utilizing antiserum adsorption techniques, should clarify the T3B-T3C antigenic picture.

No conclusions concerning the actual mechanism involved in the T3B-T3C mutation can be formed from the results of this investigation. However, certain ideas may be discussed which may influence future research concerning these phages. Considering the gross changes detected in the mutation from T3B to T3C, the gain of an additional tail antigen and possibly a qualitative change in the head antigen, one views the possibility of a point mutation with extreme skepticism.

Due to the regularity and uniformity of the mutation, a regular deletion (or addition) of a particular portion of the genetic material might seem a likely candidate. However, to gain an antigen by genetic deletion seems unlikely, unless a suppressor gene is contained within the deleted portion. Also against the deletion concept is the fact that with an antigen gain (protein) and genetic material loss (nucleic acid) the

density of T3C should be less than the density of T3B. The results obtained in this study are in exact opposition.

The fact that T3C appears slightly more dense than T3B might indicate a gain in genetic material. Thus a duplication might be involved. It would be unusual for a duplication of a portion of the phages own genome to result in the changes observed in this mutational system. Conceivably a gain in genetic material could result from genetic interaction between host and parasite. Possibly the slight density increase observed is caused by the incorporation, in rare phages, of pieces of host genome which direct the synthesis of the new phage antigens. Variation in the amount of host genome incorporated in these rare phages might account for the wider density heterogeneity of T3C suggested by the results of Plates V and VI.

It would appear that profitable information might be obtained in future studies which critically examine the nucleic acid content of these two phage types.

CONCLUSION

Purified CsCl was employed to form density gradients in investigations of bacteriophages T3B and T3C by isopycnic density gradient centrifugation. The location of the phages within the gradient was determined by infectivity titrations and the use of radioactively labeled (P^{32}) phages.

The results of one series of experiments show that T3B and T3C particles have a density, in a CsCl gradient, of 1.49 g/ml. When single drops were collected from the gradient, and analyzed for infectivity, a one drop separation in the peak titers of T3B and T3C was observed. Although the plots (infectivity titer vs. drop number) of T3B and T3C are separated

in the portion of the gradient below the band, as one would expect if a slight density difference exists, the lines fuse in the region of the gradient above the band. This observation is discussed in relation to a possible mutational mechanism.

The possibility that phage which occur outside the band in a gradient are genetic mutants was investigated and rejected. The results indicate that these phage are "unclean", and by recentrifugation can be made to band at their genetically determined density of 1.49 g/ml.

Isopycnic and sedimentation studies of deoxyribonucleic acid extracted from T3B and T3C do not reveal a dissimilarity in the phage genomes. However, more refined techniques may be successful in detecting a difference.

A new technique for antigenic analysis has been developed in this work. Phage particles combined with antibody protein are observed to be lowered in density, and in a density gradient are separated from particles which have not combined with antibodies. Application of this technique in investigations of T3B and T3C suggest that in the mutation from T3B to T3C, a qualitative change in head protein is involved, in addition to the acquisition of an additional neutralizable antigen previously described.

The results obtained in this investigation indicate that an investigation of phage-host genetic interaction might provide valuable information concerning the mechanism involved in the T3B to T3C mutation.

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DENSITY GRADIENT STUDIES OF BACTERIOPHAGE T3

by

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Variants of bacteriophage T3 have been reported that differ with regard to antigenic, morphological, and host range properties. Two of these T3 variants, designated T3B and T3C, are of particular interest. When samples are taken from individual plaques of T3B, particles of the T3C type are regularly found at a frequency of about 10^{-6} . The experiments described in this work constitute an effort to determine if T3B and T3C differ in respects not previously recognized.

The first investigations were devoted to a study of the density relationship of phages T3B and T3C. These phage were centrifuged in cesium chloride solution until an equilibrium position was attained. Fractions were collected from the gradient and analyzed for the presence of phage. Phage location was determined by infectivity titrations and in experiments where P^{32} labeled phage were employed, phage location was also determined by the presence of radioactivity in a fraction.

The deoxyribonucleic acid (DNA) of phages T3B and T3C was examined in hopes of obtaining data which would suggest a mechanism for the mutation from T3B to T3C. DNA extracted from these phage was centrifuged in cesium chloride to determine density relationship. The presence of DNA in fractions collected from the gradient was determined by absorption at $260 m\mu$ in a Beckman spectrophotometer. Sedimentation analysis of deoxyribonucleic acid from these two phage types was performed by centrifuging their DNA in a sucrose gradient. The sucrose gradient, at its point of maximum density, was less dense than the nucleic acids involved. The centrifugation process was curtailed before the nucleic acid molecules could migrate to the bottom of the centrifuge tube, fractions were collected and analyzed for the presence of DNA by absorption at $260 m\mu$.

The effect of antibodies on phage particle density was investigated. Phage were incubated in antiserum and following incubation the mixture was centrifuged in cesium chloride solution. Neutralized phage particles were located by two methods. One method was to use P^{32} labeled phage. The second method involved the digestion of antibodies by the enzyme papain, resulting in reactivation of the neutralized phage.

The results obtained indicate that T3B and T3C are slightly different in density. The peak titers of these two phage types are separated by one drop in a cesium chloride density gradient. Rare phage particles which occur at extreme positions outside the phage band in a density gradient were examined. The possibility that these phage are genetic density mutants was considered and rejected.

Density and sedimentation studies of DNA extracted from T3B and T3C did not reveal a dissimilarity in the phage genomes.

A new technique for antigenic analysis has been developed in this work. Phage particles combined with antibody protein are lowered in density, and in a density gradient are separated from phage particles which have not combined with antibodies. Using this technique data are obtained which suggest that in the mutation from T3B to T3C, a qualitative change in head protein is involved, in addition to the acquisition of an additional neutralizable antigen previously described.